In Arrayed Ranks

Array Technology in the Study of Mesothelioma

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Abstract: Mesothelioma is a rare malignancy arising from mesothelial cells lining the pleura and peritoneum. Advances in modern technology have allowed the development of array based approaches to the study of disease allowing researchers the opportunity to study many genes or proteins in a high-throughput fashion. This review describes the current knowledge surrounding array based approaches with respect to mesothelioma research.

Key Words: Mesothelioma, Array, Transcription, Proteome.

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Mesothelioma—Clinico–Pathological Features

Mesothelioma is a rare malignancy arising from mesothelial cells lining the pleura and peritoneum. For the purposes of this review we shall concentrate on the most common form of mesothelioma, malignant pleural mesothelioma (MPM). Currently rates of MPM are rising and estimates indicate that the incidence of MPM will peak within the next 10 to 15 years for the western world,[1,2] while Japanese estimates are predicted to occur for approximately 40 years.[3] Untreated, MPM has a median survival time of 6 months, and most patients die within 24 months of diagnosis.[1,4] Historically, three distinct subtypes of MPM can be distinguished based on histologic features: epithelial, sarcomatoid and mixed/biphasic. Currently, a combination of pemetrexed and cisplatin is considered to be the standard of care as a front line chemotherapy in MPM patients because it has been shown to significantly improve response rates, time to progression, overall survival, and quality of life when compared with cisplatin alone.[5,6]

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Treatment of MPM does not have one widely accepted treatment modality, which has been exacerbated by a lack of randomized clinical trials to compare available regimens.[1] Currently treatment modalities include surgery Video-Assisted Thoracoscopy, pleurectomy/decortication or extrapleural pneumonectomy, radiotherapy, or chemotherapy. For patients with earlier stages of MPM, systemic chemotherapy and more aggressive multimodal approaches, including chemotherapy, extrapleural pneumonectomy, and radiotherapy may offer more effective treatment than stand alone treatment.[6–8] However, the majority of patients with mesothelioma present with advanced disease.[6]

Array Technologies—Types, Paradigms, and Pitfalls

The advent of microarray technology has enabled researchers to examine many genes or proteins from the same sample simultaneously.[9] Other uses for microarray technology include the development of microarray-based techniques for mapping disease loci using high-density single nucleotide polymorphism (SNP)-genotyping arrays,[10,11] and comparative genomic hybridization arrays (array-CGH) to identify chromosomal losses and gains within tumors.[12]

Microarray approaches have also been used to examine the role of the regulatory chromatin environment relating to aberrant gene expression in cancer, with the development of chromatin immunoprecipitation -on-Chip arrays,[13–15] CpG island microarrays,[16] and combining epigenetic inhibitors with gene expression arrays.[17,18]

One of the most exciting uses of microarrays within the clinical setting, is their potential use as (a) prognostic factors for clinical outcome,[19–23] (b) predictors of resistance to chemotherapy,[24] and (c) in pharmacogenomics of drug response.[25] In the adjuvant setting, to avoid missing the few patients who may benefit from treatment, most cancer patients are currently overtreated. Potentially, microarray based technologies within the clinical setting could allow personalized therapies geared towards the individual and based on their molecular profiles.[26] However, a caveat to the current potential of microarray based personalized therapies is that many studies use samples which contain admixtures of stromal or other cell types in addition to the tumor cells. This can lead to complications not only in the precise delineation of gene profiling between nontumor and tumor,[27] but issues of reproducibility and accuracy can consequently cause prob-

lems with regulatory agencies.[28,29] The other main problems which have been associated with poor microarray reliability and reproducibility stem from suboptimal array design and incorrect probe annotations, which can be partly rectified through well designed analysis.[9]

The ongoing difficulties for the use of microarray technologies in the regulatory setting has resulted in the Food and Drug Administration setting up a specific portal to provide up-to-date regulatory and background information in relation to genomic studies concerning biomarker identification or pharmacogenomics.[30,31] This increasing importance for array reliability and reproducibility resulted in the setting up by the Food and Drug Administration of a Microarray Quality Control project (http://www.fda.gov/nctr/science/centers/toxicoinformatics/maqc/), the results of which have recently been published. These exciting developments indicate that both intraplatform consistency and interplatform concordance can be achieved, and represents the first important step in establishing the acceptance of microarrays for use in clinical and regulatory settings.[32,33]

In the following sections we shall describe the current use of array technology in mesothelioma research.

Transcriptomic Analysis of Mesothelioma Using Microarrays

The transcriptome can be described as "a collection of all the gene transcripts present in a given cell" and includes splice variants, and noncoding transcripts. Gene expression microarrays were developed to address how in a given population of cells a profile of the transcriptome can be identified. A significant number of studies have used gene microarray studies to examine the gene expression signature of mesothelioma. These can be subdivided into two types of study, those which involve analysis of mesothelioma derived cell lines, and those which involve mesothelioma patient samples. In the following sections we will describe the current knowledge obtained from both types of study.

Gene Expression Microarray Studies Utilizing Cell Lines

Several gene expression profiling studies have been carried out on mesothelioma derived cell lines. Rihn et al.[34] used both a cDNA microarray comprising 6969 probes and a high density filter array to compare gene expression profiles between a control SV40 transformed mesothelial cell line (Met-5A) and a mesothelioma derived cell line (MSTO-211H). The analysis identified genes which were up-regulated in MSTO-211H when compared with Met-5A. The categories of genes which were up-regulated included those involved with macromolecule stability and metabolism (e.g., heat shock proteins, *HSP60*, *HSP90*; Fatty Acid-Binding Protein 5, *FABP5*), adhesion (examples include integrin α3, *ITGA3*, integrin α4, *ITGA4*, and integrin α6, *ITGA6*), invasion (plasminogen activator inhibitor, type 2, *PAI-2*), cell cycle regulation and growth (e.g., cyclin H, *CCNH*; cyclin dependent kinase 7, *cdk-7*; V-KI-RAS2 Kirsten Rat Sarcoma Viral Oncogene Homolog, *Ki-Ras*; and the MYC oncogene, *c-MYC*), and oxidative stress response (e.g., superoxide dismutase 1, *SOD1*). Overall these differences indicate that these pathways may be important for the pathogenesis of mesothelial cell transformation, and the well documented chemo- and radio-resistance of mesothelioma.

In an array based study of four malignant mesothelioma cell lines established from primary tumors (M14K, M24K, M25K, and M38K), comparison was made with two reference primary mesothelial cell cultures derived from the pleural fluid of noncancer male patients. Up-regulated genes in the malignant mesothelioma cell lines were found to be involved with determination of cell fate (e.g., Jagged 1, *JAG1*), cell cycle (including cyclin D1, *CCND1*; cyclin D3, *CCND3*; and CDK phosphatase, *CDC25B*), cell growth (e.g., Nerve Growth Factor 2, *NgF2*; Fibroblast growth factor 3, *FGF3*), adhesion (e.g., cell adhesion molecule 1, *L1CAM*), cell motility (e.g., integrin β3, *ITGβ3*), cell invasion (e.g., keratinocyte growth factor, *FGF-7*) and DNA damage response (Inhibitor of Growth 1, *ING1/p33ING1*), indicating that these genes may be important for MPM tumor invasion.[35]

Using a cDNA microarray approach to identify genes which were overexpressed in a panel of seven mesothelioma cell lines, Kim et al.[36] identified a regulator of microtubule dynamics, stathmin, as being a gene which is frequently up-regulated in these cell lines, and subsequently demonstrated its overexpression in primary malignant mesothelioma samples. Aberrant microtubule dynamics caused by the overexpression of stathmin may play important roles in the pathogenesis of mesothelioma.

Effects of Fibers/Asbestos on Gene Expression Profiles in Mesothelioma Derived Cell Lines

As asbestos exposure has been clearly implicated in the development of MPM, several studies have attempted to document the changes in gene expression in mesothelial and mesothelioma cell lines exposed to asbestos fibers. In a study of rat pleural mesothelial cells exposed to asbestos, genes that were up-regulated were found to mostly function in cellular transport, but protein kinases and proto-oncogenes such as hepatocyte growth factor receptor (c-Met), c-myc and fos-related antigen-1 (*Fra-1*), were also identified as being significantly up-regulated. *Fra-1* expression has been previously shown to be a critical to the morphologic transformation of mesothelial cells.[37]

A similar more recent study by Nymark et al., examined the response of Met5A to crocidolite asbestos exposure. Using Gene Ontology analysis, the authors identified several important Gene Ontology biologic processes for which gene expression was either up- or down-regulated following exposure to asbestos fibers. These included the down-regulation of genes associated with cytoskeletal anchoring, while genes involved with nucleosome assembly, regulation of translational initiation, and the regulation of cell survival were found to be up-regulated.[38] The pathways identified by Nymark et al., may therefore be also important pathways activated by asbestos in the pleura, and as such may play important roles in the progression to MPM.

In a microarray study on Met-5A mesothelioma cells exposed to crocidolite, erionite, and the proinflammatory cytokines IL-1Beta and TNF-alpha, Swain et al.,[39,40] demonstrated that the chemokine superfamily including the chemo-

kines CXCL1 (GROα), CXCL10, IL-8, and CCL20 were significantly up-regulated following exposure to these conditions. Independent confirmation for the up-regulation of CXCL10 and IL-8 has been observed in mesothelioma cell lines,[41–43] although one study observed up-regulation of IL-10 shortly after exposure to crocidolite, but which was subsequently lost over long-term exposure.[38]

Chemokines are chemoattractant cytokines which function to regulate the trafficking and activation of leukocytes and other cell types under a variety of inflammatory and noninflammatory conditions. Increasingly, studies have shown that chemokines play an important role in several aspects of tumor progression.[44,45] Tumor cells express functional chemokine receptors, which can sustain proliferation, and survival and promote organ-specific localization of distant metastases. Furthermore, chemokine expression in tumors is also associated with hindering antitumor immune responses through regulation of leukocyte infiltration and thereby favoring the establishment of immune escape mechanisms in the tumors.[46] In a seminal study, elevated expression of IL-8 was observed in pleural fluid samples from mesothelioma patients, and immunohistochemistry of pleural biopsy specimens showed that IL-8 was localized to the malignant mesothelioma cells, but not to other types of malignant metastatic deposits. In vitro mesothelioma cell lines were subsequently shown to constitutively express IL-8 mRNA while normal resting human mesothelial cells did not.[47] While IL-8 is known to have angiogenic activity,[48] it has also been shown to have growth-promoting activity in mesothelioma and inhibition of IL-8 activity in a nude mouse model of mesothelioma resulted in a reduction of MPM progression.[49,50] Indeed, in lung and colon cancer cells Toll-Like Receptor 4 (TLR-4) signaling has been shown to promote immune escape by inducing both IL-8 and CCL20.[51,52] This observation coupled to the data showing that Met-5A MPM cells stimulated with TNF-α or IL-1β overexpress these chemokines provides a potential link between chronic inflammation and tumor immune escape in mesothelioma, and indicate that IL-8 may represent a therapeutic target in the treatment of mesothelioma.

The up-regulation of the chemokine superfamily in mesothelioma cells exposed to fibers therefore represents a critical pathway in malignant mesothelioma pathogenesis, linking inflammation, angiogenesis, and tumor immune escape.

Changes in Microarray Gene Expression Profiles in Mesothelioma Cells Undergoing Differentiation

Malignant pleural mesothelioma is an aggressive tumor, characterized by a variable differentiation pattern. Clinical outcome in patients relies mainly on predictions based on the morphologic phenotype of the tumor. In an attempt to study MPM tumor differentiation, Dobra and coworkers used an array approach to examine an in vitro model of MPM differentiation, by studying the gene expression profiles of two well established MPM cell sublines. The authors compared the gene expression signature of the epitheliod STAV-AB MPM cell line to that of the Fibroblastoid STAV-FCS cell line. Two expression pattern signatures (comprising a total of 102 up-regulated genes) were identified which could distinguish between epithelial and sarcomatoid subtypes. Sarcomatoid tumors contained elevated levels of growth factor receptors and associated binding proteins (examples include Platelet-Derived Growth Factor Receptor Beta, PDGFRβ; Fibroblast Growth Factor Receptor 1, FGFR1; Transforming Growth Factor beta 1, *TGFβ1*), whereas epithelial mesothelioma cells were found to have overrepresentation of tumor promoting factors involved with differentiation (examples include Keratin 5, *KRT5*; Bone morphogenetic protein 4, *BMP4*), metabolism (Phosphatidylinositolglycan class F, *PIGF*; Proteasome activator subunit 2, *PSME2*), and regulation of apoptosis (Immediate early response 3, IER3; Nuclear factor of kappa light polypeptide gene enhancer in B-cell inhibitor, alpha, *NFKBIA*).[53] Overall, the expression profile for the epitheliod cell line was that expected for a more differentiated tumor. In MPM epitheliod histology is associated with a lower malignant potential and better responses to therapy. The fibroblast-like cell line had a profile more commonly associated with growth factors and genes that may contribute to the particularly unfavorable prognosis of sarcomatoid tumors.

In a similar study Hida et al., established two new MPM cell lines, Y-MESO-8A (epithelial-like morphology) and Y-MESO-8D (spindle-like morphology) from a patient with biphasic (sarcomatoid/epithelial) features. Using microarray gene expression based profiling to compare between these two cell lines, the authors found 43 genes which had greater than fivefold differences between the two cell lines. Genes involved with cell structural activity (e.g., Filaggrin, *FLG*; Microfibril-associated glycoprotein-2, *MAGP2*) or cell adhesion (e.g., Tissue inhibitor of metalloproteinase 3, *TIMP3*) were overexpressed in the epithelial (Y-MESO-8A) cell line over the spindle (Y-MESO-8D) cell line, but no differences in the expression of major cancer associated genes were found between the two cell lines.[43] In MPM, it is generally considered that the sarcomatoid (spindle-like) subtype is a more aggressive form than the epitheliod subtype.[43] In the same study, 15 genes were identified which had greater than fivefold up-regulated expression in the sarcomatoid subtype (Y-MESO-8D) compared with the epithelial like subtype (Y-MESO-8A) were identified.[43] These included the proinflammatory cytokines interleukin-1α (*IL-1α*), and interleukin 8 (*IL-8*), and several genes involved with cell growth and communication (including Vascular cell adhesion molecule 1, *VCAM1*; and Solute carrier family 21 (organic anion transporter), member 9, *SLC21A9*). Indeed in a small study of 16 cases, immunohistochemical analysis demonstrated VCAM-1 expression in 14 of 16 cases of mesothelioma.[54] In a lung epithelial cell culture model IL-1alpha has been shown to be able to regulate expression of VCAM-1,[55] and in an early study in melanoma, cells overexpressing IL-1alpha when injected into mice, increased the expression of VCAM-1 on lung microvascular endothelial cells.[56]

While the function of *SLC21A9* has been proposed to be involved with the uptake of steroid hormones and their conjugates, drugs, and numerous anionic endogenous sub-

strates,[57] SLC21A9 also shares 42.8% amino acid sequence identity with a rat prostaglandin transporter.[58] As prostaglandins are linked to both inflammation and cancer, the data obtained from the array study supports the idea that in spindle cells there is a more proinflammatory environment resulting in elevated expression of adhesion molecules such as VCAM1, and may therefore explain to some degree why in the spindle subtype of MPM is associated with a more aggressive phenotype.

Gene Expression Profiling of Primary Mesothelioma to Identify Predictive or Prognostic Biomarkers

With the advent of gene expression profiling frequent use of arrays has been carried out on MPM patient material, in attempts to identify profiles which may be predictive (predictive of disease presence), prognostic (predictive of long term patient prognosis) and/or treatment response (predictive of personalized therapy).

Bueno and coworkers initially used arrays to distinguish MPM from lung cancer through differential gene ratios.[59] A subsequent follow-up study identified 46 candidate prognostic genes for MPM and defined a four gene set whose expression ratios could predict treatment related outcome.[60] More recently, the authors validated this signature in a further study of MPM, and identified seven new potential prognostic markers.[61] In a similar experiment Pass et al.,[62] using neural network classification, identified a 27 gene signature for MPM which was both prognostic for patient survival and predictive for disease progression. Using Onto-Express analysis, a tool which can automatically translate lists of differentially regulated genes into functional profiles for biochemical function, biologic process, cellular role, cellular component, molecular function and chromosome location,[63] the authors identified the following biologic processes impacted by their 27 gene signature: cell proliferation, lipid metabolism, positive regulation of cell proliferation, regulation of transcription from Pol II promoters, pathogenesis, cell cycle arrest, negative regulation of cell proliferation, inflammatory responses, induction of apoptosis by extracellular signals, DNA damage response through activation of p53, and RNA processing.[62]

A study from the group of Marc Ladanyi on MPM also identified a 29 gene signature set which had prognostic value, and compared their results to those of Bueno and Pass. Their conclusion was that gene expression profiling had an upper limit of around 65% predictive, below the level of clinical usefulness.[64] Nevertheless, the authors identified gene Aurora Kinase B, AURKB, which was overexpressed in both sarcomatoid and epithelial MPMs. However, the overexpression was only associated with unfavorable outcome in the epithelial subtype.[64] Using Ingenuity Pathway Analysis software, the authors linked 11 of their 29 gene prognostic classifier set into a single network of genes associated with cell cycle control and mitosis, cell death and cancer.

Glinsky et al.[65] have identified an 11 gene stem cell like signature driven by the Leukemia Viral Oncogene BMI-1, which has prognostic power in MPM, and 10 other distinct types of cancer. This 11 gene signature was associated with (a) highly malignant cancers, (b) propensity toward metastatic dissemination, and (c) with predicting a high probability of therapy failure in these cancers. These results may consequently have general application in the diagnosis and management of cancer, and may prove to be an important breakthrough in understanding cancer biology. One caveat in relation to the results obtained for MPM, is that the number of samples involved was small ($n = 17$), and so independent validation of these results in a large prospective study are warranted.

Gene Expression Profiling Studies to Identify Mesothelioma Biomarkers/Pathways

In a study examining 10 MPM cell lines and four primary tumors Kratzke and coworkers[66] used gene expression profiling to identify a subset of 180 genes whose differential expression had the capability to distinguish between MPM subtypes. This study identified matriptase (ST14) as a gene which differentiated the epithelial subtype from sarcomatoid or biphasic MPMs, and identified insulin-like growth factor binding protein 5 (*IGFBP*-5), as a gene significantly down-regulated in MPM.

Other groups have attempted to use gene expression microarrays to identify potential MPM biomarkers, or important pathways which may be important for MPM pathogenesis. One early study by Albeda and coworkers[67] revealed that pathways involved with glucose metabolism, protein translation and cytoskeletal remodelling were frequently altered in MPM. Using a combination of laser capture microdissection and gene expression arrays Rihn and coworkers[68] identified 14 genes. Of these eight were up-regulated (complement factor b, *BF*; ferritin light polypeptide, *FTL*; Insulin-like growth factor binding protein 7, *IGFBP7*; retinoic acid receptor responder 1 *RARRES1*; retinoic acid receptor responder 2, *RARRES2*, retinol-binding protein 1, *RBP1*; spermidine/spermine N1-acetyltransferase, *SAT*; Thioredoxin, *TXN*), while six were down-regulated (arachidonate 5-lipoxygenase-activating protein, *ALOX5AP*; chloride channel nucleotide-sensitive 1A, *CLNS1A*; eukaryotic translation initiation factor 4A, isoform 2 *EIF4A2*; ETS-Domain Protein 3, *ELK3*; REQUIEM, Apoptois Response Zinc Finger Gene, *REQ*; and synaptophysin-like protein, *SYPL*) in microdissected MPM cells compared with microdissected mesothelial pleural cells.

Mohr et al., identified 700 genes in two primary epithelial MPM tumors which were differentially expressed compared with a pleural cell line (MET-5A). Two major categories of genes identified were found to be altered in MPM. The first, (cell protection and resistance), included subcategories involving nucleic acid integrity and repair; protein stability and maintenance, oxidative stress and drug metabolism. The second category (Tumor invasiveness), included subcategories such as adhesion, extra cellular matrix components, and genes involved with metastatic potential.[69]

In a study examining the gene expression profiles of 17 mesothelioma patients with different overall survival times, Gordon et al.,[60] defined 46 potential prognostic molecular markers which could distinguish two outcome-related groups of patients. Taking the top four genes which were the most statistically significantly overexpressed in each outcome

group, by examining the various gene expression ratio permutations they narrowed this set down to four gene pair ratios whose expression ratios could predict outcome. The genes identified using this strategy were Cordon-bleu protein-like 1, *COBL-like 1*/*KIAA0977*; cytosolic thyroid hormone binding protein, *CTHBP*, Guanosine diphosphate-dissociation inhibitor 1, GDIA1; and an EST similar to the L6 tumor antigen),[60] and this prognostic test was subsequently validated in a follow-up study.[61]

Using a different microarray platform and 39 different MPM samples Gordon et al., identified seven new candidate prognostic markers, whose expression ratios could stratify patients with poor outcome from those with better outcome. The identified genes were CD9 antigen, *CD9*; Discs, large (Drosophila homologue) 5 *DLG5*; complement component 3, *C3*, and one *EST*; DKFZp586J2118 with unknown function, small cell lung cancer carcinoma cluster four antigen, *CD24*; *KIAA1199*; and thrombomodulin, *THBD* in MPM.[61]

Bueno and coworkers[70] also used microarray gene expression profiles to identify 328 genes significantly up-regulated in MPM tumors relative to normal tissues. Unsupervised clustering of these genes identified two potential subclasses of mesothelioma- that correlated loosely with tumor histology. Further analysis of this clustering identified sets of genes which could distinguish between multiple tumor subclasses, normal and tumor tissues, and tumors with different morphologies. Three novel up-regulated candidate oncogenes (nucleotide-diphosphate kinase 2, *NME2*; CREBBP/EP3000 inhibitory protein 1, *CRI1*; Platelet-derived growth factor C, *PDGFC*) and one novel down-regulated candidate tumor suppressor gene (Gelsolin, *GSN*) were identified in this analysis.[70]

Knuutila and coworkers[42] examined the expression of 588 genes associated with cancer in MPM, and identified 25 genes which were deregulated. Examples of the genes identified included down-regulation of the growth factor (Basic Fibroblast Growth Factor, *BFGF*), elevated expression of the chemokines (CXCL10, and CXCL2) and both elevated and reduced expression of genes involved with cell adhesion. Ezrin was found to be reduced while Integrin β4 levels were elevated. Genes involved with tumor invasion (Matrix metalloproteinase 9, *MMP9*) were found to be elevated, and also proto-oncogenes (colony-stimulating factor-1 receptor, *CSF1R*). A multidrug resistance gene (Semaphorin 3C, *SEMA3C*), was also found to be overexpressed in these samples. Overexpression of this gene in recurrent lung carcinomas and in a cisplatin resistant MPM line is associated with drug resistance.[71]

Another potential therapeutic pathway for MPM was discovered using a custom array designed to examine the *Wnt* signaling pathway which identified *Wnt2* as being a potential new biomarker for mesothelioma.[72]

Inhibitor of apoptosis-1 (IAP-1), was previously been shown to be a novel up-regulated gene in MPM using differential display.[73] In a follow-up study Gordon and coworkers used gene expression profiling to examine the expression of IAP family members in MPM, and demonstrated that several family members had prognostic value. *IAP-1* and Baculoviral IAP Repeat-Containing Protein 5 (*BIRC5*, also known as *Survivin*) expression correlated with a relatively shorter patient survival, while Inhibitor of apoptosis, X-linked, (*XIAP*) and Baculoviral IAP Repeat-Containing Protein 7 (*BIRC7*, also known as *livin*) were associated with longer patient survival.[74]

Gene Expression Profiling to Examine Response to Therapy in Mesothelioma

Gene expression profiling has also been used to examine the response to therapeutic drugs in mesotheliomas. In a phase I clinical trial for the effects of Decitabine (a DNA methyltransferase inhibitor) in patients with cancers of the lung, esophagus or pleura, David Schrump and coworkers examined tumor biopsies pre and posttreatment for target genes (NY-ESO-1, MAGE-3, and p16), known to be down-regulated by methylation. They demonstrated robust alterations to their expression in 8 of 22 individuals which completed treatment. Subsequently, using laser capture microdissection of tumor cells isolated from one MPM patient before and after decitabine treatment, they observed that prolonged low-level decitabine exposure could modulate global gene expression patterns (75 genes induced, 324 genes repressed $\geq$twofold by decitabine) in this patient.[75] Of these pathways, members of the Notch signaling, Heterotrimeric G-protein signaling (Gq alpha and Go alpha mediated & Gi alpha and Gs alpha mediated pathway), JAK/STAT signaling, Interferon-gamma signaling, and chemokine and cytokine signaling inflammatory pathways were identified as having originally been up-regulated or overexpressed in the tumors but subsequently down-regulated following decitabine treatment ([75]supplementary information Table 1).

Using Arrays to Identify Cytogenetic Alterations in Mesothelioma

Standard techniques for the identification of chromosomal abnormalities (Karyotyping, CGH) have confirmed that there are frequent complex chromosomal imbalances

TABLE 1. Predictive Genes in MPM for Which Alternative Validation Exists

Gene	Original Array Study	Confirmation Array Study
BIRC1	64	74,151
CRIP1	62	70
HEG1	62	70
IGFBP-5	62	42,70[a]
MYC	62	70
PLXNA3	64	42
RARRES1	64	68,70
SEMA3A	60	70
SPOCK2	62	67,70
WT1	64	70

[a] Caveat–IGFBP-5 was found to be commonly down-regulated by Hoang CD, D'Cunha J, Kratzke MG, et al. Gene expression profiling identifies matriptase overexpression in malignant mesothelioma. *Chest* 2004;125:1843–1852.

BIRC1, Baculoviral IAP repeat-containing protein 1; CRIP1, cysteine-rich intestinal protein 1; HEG1, heg homolog 1 (zebrafish); IGFBP-5, insulin-like growth factor binding protein 5; MYC, v-myc avian myelocytomatosis viral oncogene homolog; PLXNA3, plexin A3; RARRES1, retinoic acid receptor responder 1; SEMA3A, Semaphorin 3A; SPOCK2, sparc/osteonectin, cwcv, and kazal-like domains proteoglycan 2; WT1, WT1 gene.

associated with MPM.[76] These cytogenetic changes, however, are limited in relation to their mapping resolution. Array technology has the potential to more precisely demarcate cytogenetic abnormalities in MPM, with the development of both CGH-Arrays and SNP-based mapping arrays. Several recent reports have used these technologies in the study of MPM, and the main findings have been summarized in Table 2.

One of the limitations of standard cytogenetic methods is that resolution, in relation to the precise location of the cytogenetic changes is poor. Currently, standard CGH can provide only limited resolution at the 5 to 10 Mb level for the detection of copy number losses and gains, and at 2Mb for amplifications. Array-CGH has overcome some of these limitations bringing resolution down to approximately 0.7 Mb. High density oligonucleotide microarrays based on SNP have recently been shown to identify both copy number alterations and loss of heterozygosity at very high resolution.[77–79] Using this approach Testa and coworkers examined loss of chromosome 9p21 (a region which contains CDKN2A also known as

TABLE 2. Most Frequent Copy Number Alterations Found in Mesothelioma of Chromosomal Losses and Gains of Malignant Mesotheliomas as Detected by CGH, Array-CGH or SNP-Genotyping

Standard Cytogenetics (Cytogenetics, FISH, LOH)	CGH	Array-CGH	SNP-Array	Potential Important Genes Implicated in MPM
Gains				
+1, +3, +5, +6, +7, +11, +17, +20	1p31.3-pter, 1q (incl. 1q23, 1q32,) 4p15.3-pter, 5p, 5q31-qter, 6p, 7, 7p14-p15, 7pter-q11.2, 7q22, 7q32-q33, 8pcen-p12, 8q, 8q22–23, 8q24-qter, 10q25.1-qter, 11qcen-q22, 12, 13q13.3-q14.2, 14q24-qter, 15q22-qter, 17q22-q24, 20, 22, 22q trisomy 19, trisomy 22q	1p32.1, 1q, 2p (incl. 2p11.2-q11.2, 2p14.3-p13.3, 2p23, 2p25.1, 2p23), 2q32.1, 3q11-q27, 4p12, 4p13, 4p14, 4p15.1, 4p16.3, 5p (incl. 5p15.33-p13.1), 7p (incl. 7p15.1, 7p22.3-p22.2), 8q24, 8q24.3, 9p13.3, 9q, 10q24.1-q24.2, 10q26.12-q26.13, 10q26.13, 11p15.5-p15.4, 11p15.4, 11q12.1-q13.1, 11q22.1, 11q22.2-q22.1, 12p13.2-p13.1, 12q13.3, 15q11.2-q12, 17q (incl. 17q21.32-qter, 17q21.31, 17q21.33), 19p13.12, 20p, 20p11.21-q11.21, 20q11.22-q11.23, 20q13.12, 20q13.13, 21q11.2, Xp22.33, Xq28		1p32.1 (*JUN*) 11q13 (*Fra-1*) 11q22 (*YAP1*, *CHK1*)
Losses				
1p21-p22, 3p21, 3p21.3, 4p, 4p15, 4q, 4q25-q34, 6q (incl. 6q14-q25, 6q15-q21, 9p (incl. 9p13, 9p21, 9p22, 9p21-p22), 11p11-p13, 13q, 13q 13.2-q14.2, 14q, 15q15, 17p13, 22, 22q12	1p, 1p11-p22/p31, 1p21, 3p, 3p21, 4p (incl. 4p11-p13/p15, 4p12-p13), 4q31.1-qter, 4q31-q22, 5q, 6q (incl. 6q11-q21, 6q12-q14, 6q14, 6q22-q24), 7q, 8p (incl. 8p12-p21, 8p21-pter), 9p (incl. 9p13-p21, 9p21, 9p21-pter), 10p13-pter, 13 (incl. 13q12-q14, 13q13-q14, 13q21.1-q22), 14, 14q (incl.14q12-q24, 14q24.2-qter), 15, 15q (incl. 15q11.1-q15, 15q11.1-q21, 15q11.2–13, 15q12–14; 15q14-q15), 17p, 17p12-pter, 22, 22q, Y	1p (incl. 1p13.1p12, 1p31.1, 1p31.1-p13.2, 1p36.33, 1p36.1, 1p21.3), 1q34-qter, 3p (incl. 3p22.1-p14.2, 3p21.2–21.3), 3q27-qter, 3q27.3-q28, 4, 4q (incl. 4q22, 4q34.3-q35.1, 4q34-qter), 6p11.2-q12, 6q (incl. 6q12-q16.3, 6q22.1, 6q22.31, 6q25, 6q26), 7q35, 9p (incl. 9p21.1, 9p21.2, 9p21.3, 9p24.3-p21.2), 10p (incl. 10pter-p12.1, 10pter-p11.22, 10p11.22, 10p11.21, 10p15.3), 10q26.2–10q26.3, 11q, 11q25, 13, 13q (incl. 13cen-q14.12, 13q33.2), 14, 14 q (incl. 14q22.1-qter, 14q32.13), 15q15.1-q21.1, 16q, 18, 18q, 22q (incl. 22q11.1-q11.23, 22q11.23-q13.33, 22q12.2, 22q12.2-q12.3, 22q12.3, 22qcen-q12.3), Xq25, Y	9p21	3p21 (*RASSF1A, CTNNB1*) 9p21 (*CDKN2A/ARF, p16INK4a*) 17p13 (*TP53*) 22 (*NF2/Merlin*)

Identified from Pubmed using the following search expressions: ″mesothelioma CGH,″ ″mesothelioma array-CGH″ ″mesothelioma array-based comparative genomic hybridization″ ″mesothelioma SNP array,″ ″mesothelioma cytogenetics.″

Additional information came from http://www.mesotheliomainfected.com/cytogenetics-of-malignant-mesothelioma.html.

LOH, loss of heterozygosity; FISH, fluorescence in situ hybridization; CGH, comparative genomic hybridization arrays; SNP, single nucleotide polymorphism; *JUN,* v-jun avian sarcoma virus 17 oncogene homolog; *FRA1*, fos-related antigen 1; *YAP1*, yes-associated protein 1, 65-kd; *CHK1,* cell cycle checkpoint kinase; *RASSF1A,* ras association domain family protein 1; *CTNNB1,* catenin, beta-1; *CDKN2A/ARF,* cyclin-dependent kinase inhibitor 2a (arf included); *TP53,* tumor protein p53; *NF2* (*Merlin*), neurofibromin 2.

INK4A/ARF) in 4 MPM cell lines and precisely demarcated two different, but overlapping deletions. All of the deletions in the cell lines encompassed the CDKN2A and CDKN2B loci. The four MPM cells all exhibited a similar deletion pattern; that is, each showed both a pronounced loss of signal for multiple contiguous markers in 9p21 surrounded by a larger region with a lesser loss of signal. This is considered to be indicative of a homozygous deletion embedded within a heterozygous deletion.[80] Using CGH based arrays Szlosarek et al. examined seven mesothelioma cell lines for loss of 9q34, a region which contains a gene down-regulated in MPM, argininosuccinate synthetase. The authors found no loss of heterozygosity in the cells, and subsequently went on to show that the down-regulation of this gene was through epigenetic inactivation.[81] Sekido and coworkers used genome wide array based CGH on 17 primary tumors and nine cell lines confirming the frequent losses and gains observed through standard cytogenetic or CGH methods, but also identifying new regions of interest including gains of 8q24 and loss of 13q33.2. In addition, the authors identified a high copy number gain of chromosomal region 1p32 (a region which includes the proto-oncogene *JUN*.[82]

The effects of DNA copy-number changes on MPM gene expression profiles has recently been determined using a combination of array-CGH and array gene expression profiling. Using two cytogenetically unstable MPM cell lines (PMR-MM2 and PMR-MM7) as their in vitro model, Looijenga and coworkers studied the impact of DNA copy number changes on the cells gene expression profiles over time. DNA losses and gains were identified using microarray-based array-CGH, and coupled with minimal overlapping analysis this led to the identification of several common unbalanced genomic regions at early passages with 27 regions of DNA gain and 14 regions of DNA loss. Many of these loses and gains correlate well with previously published cytogenetic studies. Following long term culture of PMR-MM7, gains of chromosomal regions 1p34.2-p34.1, 3p25.1, 16q23.2-q23.3, and 19p13.13 were observed, while loss of 7p13 occurred. Subsequently the authors analyzed the gene expression profile of one of these cell lines PMR-MM7 after early and late passages, and correlated the differentially expressed genes with the copy-number changes identified using the array-CGH. From this analysis the authors demonstrate that prolonged culture of this unstable MPM cell line led to the acquisition of additional chromosomal copy-number changes associated with dysregulation of genes involved in cell adhesion, regulation of mitotic cell cycle, signal transduction, carbohydrate metabolism, motor activity, glycosaminoglycan biosynthesis, protein binding activity, lipid transport, ATP synthesis, and methyltransferase activity.[83]

Using array-CGH on a series of 26 well characterized MPM samples, Knuutila and coworkers found that gene losses were predominant in MPM. The most frequent losses observed occurred in 1p31.1 → p13.2, 3p22.1 → p14.2, 6q22.1, 9p21.3, 13cen → q14.12, 14q22.1 → qter, and 22qcen → q12.3. Novel findings included gains of 9p13.3, 7p22.3 → p22.2, 12q13.3, and 17q21.32 → qter.[38] The most frequent region of loss 9p21.3 (occurring in 17 of the 26 MPM), contains the loci for CDKN2A and CDKN2B, two important genes known to be frequently lost in MPM.

Mesothelioma Tissue Microarrays (TMAs)

Tissue microarrays (TMAs) represent a high-throughput method for the investigation of biomarkers in multiple tissue specimens at once. Composed of arrays of core biopsies obtained from paraffin-embedded tissues, up to 1000 tissue samples can be analyzed in a single experiment using either immunohistochemistry or in situ hybridization. TMAs play a central role in translational research, facilitating the analysis of molecules that have potential roles in the diagnosis, prognosis, and prediction of response to therapy.[84–87] The advantages of TMAs include conservation of rare clinical resources, experimental uniformity/standardization, decreased assay volume, and high-throughput.[84,88] This is critically important for tumors such as mesothelioma, and allows the use of stored archival tissue.

One of the most frequent concerns surrounding TMAs, has been whether the small size of the tissue cores used can be representative of an entire tumor. Many studies have sought to address this, and have achieved high concordance. Additional studies to identify the optimal number of cores required for concordance and have found that only two-three such cores are required per sample.[85,86] There are some drawbacks to TMAs. The first is the limitation imposed by availability of validated antibodies suitable for use in immunohistochemistry. Additionally, the issue of loss of antigenicity in samples must also be considered, although this would appear to be related to how samples or slides are stored rather than in the construction of TMAs.[88] One of the biggest hindrances currently with the use of TMAs in a high-throughput setting has been TMA reading and analysis.[85,86] However, as with all such technology sophisticated hardware and software tools are emerging to alleviate this.

Several recent studies on mesothelioma have used TMA technology to study either individual protein or protein signatures in mesothelioma. Kettunen et al. identified an 11 gene signature which could differentiate between the various mesothelioma subtypes. Using TMA, the authors studied five members of this signature in more detail, and found that three of the identified genes (P-cadherin, neural cell adhesion molecule L1/L1CAM, and integrin β4/ITGB4) were significantly elevated in the epithelial subtype.[42] In a similar type of analysis Gordon et al. identified gene signatures which could be loosely correlated with mesothelioma subsets on the basis of morphology. They subsequently studied the expression of three candidate oncogenes (Nonmetastatic Cells 2/*NME2*, CREBBP/EP300 Inhibitory Protein 1/*CRI1*, and Platelet Derived Growth Factor-C/*PDGFC*), and one candidate tumor suppressor (Gelsolin/*GSN*) using TMA. Using this approach, the authors found that two of the examined candidates CRI1 and NME2 had significantly elevated expression in tumor cells, with no detectable expression of these proteins in normal pleura or stromal cells within the tumors.[70] In a follow-up study Gordon et al.[89] studied expression of the Inhibitor of Apoptosis (IAPs) family and demonstrated abundant expression of all IAPs in MPM tumors, and could be associated with MPM survival. Pass et al., used an MPM

TMA derived from 38 individuals to examine osteopontin expression. Positive staining for osteopontin was observed for 36 of the 38 individuals,[90] and subsequently serum levels of osteopontin were examined and found to have prognostic significance in the detection of mesothelioma (discussed in more detail in the following section). The c-MET proto-oncogene has been shown to be highly expressed in MPM both by standard assays and gene expression profiles.[70] More recently a study used TMA to examine c-MET in more detail, and has confirmed that this gene has elevated expression in MPM and no staining in normal pleura.[91] Szlosarek et al.[81] identified Argininosuccinate Synthetase/AS (a key regulator of the arginine biosynthesis pathway) as a gene which is frequently down-regulated in MPM, and used a TMA array to confirm that levels of AS are absent in 63% of MPM patients regardless of subtype, although predictive or prognostic values were associated with this decrease.

In a recent study for alterations of expression of Phosphatase and Tenisn Homolog, Weder and coworkers used a 341 MPM TMA. From their analysis a significantly longer median survival time occurred in patients expressing Phosphatase and Tenisn Homolog independently of histologic subtype.[92]

Array Technology to Study Mesothelioma Serum/Proteomics

Proteomics, or the ability to study all of the proteins encoded by the genome represents a branch of research with significant potential within diagnostic, screening or prognostic settings in cancer management. Within oncology, proteomics may prove to have significant benefit in the areas related to biomarker discovery, treatment, and early detection through serum screening or tissue sample analysis, and to develop targeted pharmacoproteomic therapies.[93]

Oncoproteomics has been coined to describe the idea that certain protein signatures or patterns can be associated with a particular malignancy, which if combined with clinical correlations could allow the prediction of disease progression and perhaps improved therapeutic modalities.[94] In this regard, antibody arrays allow the simultaneous measurement of many proteins in experimental samples, and show great promise in the development of high-throughput strategies for clinical proteomics in the identification of cancer biomarkers, diagnosis, and management of cancer.[95–97] Tumor-associated antigen arrays (TAAs) are also emerging as a novel approach to serological diagnosis of cancer. TAA arrays comprising several antigens against cancer-associated autoantibodies greatly increase the diagnostic utility of TAAs in the diagnosis of cancer from sera.[98]

As this area of research is still in its infancy, relatively few large scale proteomic studies on mesothelioma have been described. The most comprehensive study involved the analysis of exosomes secreted from primary mesothelioma cell lines derived from patients.[99] Of the proteins identified, several were involved with antigen presentation, signal transduction, migration and adhesion. A follow-up study on exosomes isolated from the pleural fluid of mesothelioma patients identified proteins involved with blood coagulation (Complement factors & Fibrinogen), various immunoglobulins, and Cytoplasmic linker protein 2 (KIAA0291), a protein whose function is proposed to mediate the interaction between specific membranous organelles and microtubules.[100]

Recently, using an array based technology, the expression profiles of 80 cytokines and chemokines were examined in mesothelioma cell lines and the pleural fluids obtained from the original patients from which the mesothelioma cell lines were derived.[41] Several important proteins involved with immune suppression, angiogenesis, and plasma extravasation could be detected in both cell line supernatant and primary pleural effusions including angiogenin, vascular endothelial growth factor, transforming growth factor-β, and epithelial neutrophil-activating protein-78. From the same study, immunohistological staining demonstrated heavy infiltration by many immune effector cells, and significant amounts of Foxp3 + CD4 + CD25 + regulatory T-cells, which when depleted in a murine mesothelial model increased long term survival.[41]

A significant number of studies have focused on developing serum based assays for the diagnosis of mesothelioma. The best characterized of these is the serum marker mesothelin. Soluble mesothelin (SMRP) is a 40 kDa glycoprotein cleavage product of the membrane bound form of mesothelin, which is released into plasma serum by the activities of furin-like proteases.[101] The first report that this soluble protein could be a potential useful biomarker in the detection of mesothelioma came from the laboratory of Bruce Robinson, where 84% (37/44) patients had elevated SMRP levels.[102] This sensitivity was confirmed in a follow-up study which also demonstrated 95% specificity for the enzyme-linked immunosorbent assay (ELISA) based assay.[103] Independent studies have continued to confirm the sensitivity of mesothelin.[104–107] However, a recent study has raised the issue of high false positive rates in a study comparing healthy individuals to healthy individuals exposed to asbestos.[108]

The reliability and sensitivity of the mesothelin assay has led to the development and marketing of ELISA based assays for the detection of SMRP.[78,109–111] Most recently, a phase I clinical trial targeting mesothelin with a recombinant mesothelin immunotoxin (SS1P) has been completed. Of the 33 evaluable patients treated, 4 had minor responses, 19 had stable disease, and 10 had progressive disease. A phase II clinical trial is currently being planned to expand these studies.[112] Other phase I trials targeting mesothelin involving either a chimeric antimesothelin monoclonal antibody (MORAb-009), or a live-attenuated Listeria monocytogene vector encoding human mesothelin (CRS-207) are currently running.[113]

Other serum markers identified recently include serum circulating hepatocyte growth factor,[91] osteopontin,[90] and simian virus 40 (SV40) T-antigen.[114] Serum osteopontin would appear to be somewhat controversial as the initial study did not take into account the fact that osteopontin is cleaved by thrombin in the blood,[101] and a recent study in head and neck cancer has demonstrated that different ELISA systems used to detect osteopontin can give varying sensitivity.[115] Other potential candidates emerging for the early detection of mesothelioma identified hepatocyte growth fac-

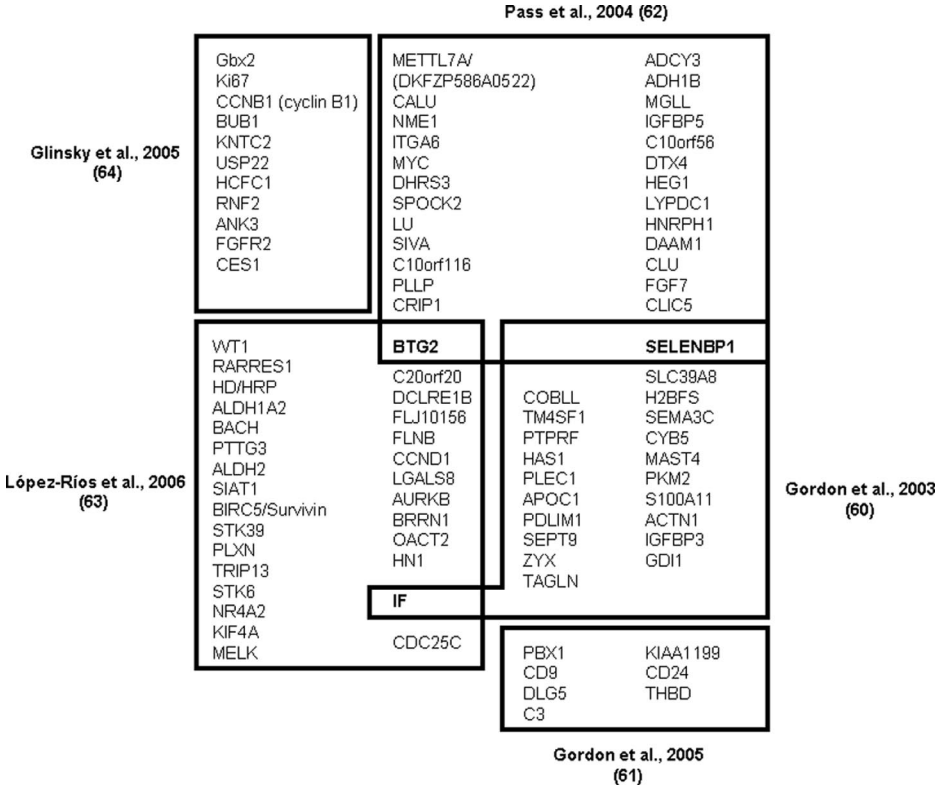

FIGURE 1. Graphical comparison of five microarray studies of malignant pleural mesothelioma (MPM)[60–62,64,65] with prognostic gene lists. Adapted with permission from López-Ríos F, Chuai S, Flores R, Shimizu S, Ohno T, Wakahara K, Illei PB, Hussain S, Krug L, Zakowski MF, Rusch V, Olshen AB, Ladanyi M. Global gene expression profiling of pleural mesotheliomas: overexpression of aurora kinases and P16/CDKN2A deletion as prognostic factors and critical evaluation of microarray-based prognostic prediction. HYPERLINK "javascript:AL_get(this,%20'jour', %20'Cancer%20Res.');" Cancer Res. 2006 Mar 15;66(6):2970–9, Figure 3.

tor, basic fibroblast growth factor, and VEGF-beta as serum markers which can significantly differentiate high-risk individuals from healthy and MPM groups. The same study found that a combination of 8-hyroxy-2-deoxyguanosine (80HdG), VEGF-beta, and SMRP as a combination of serum markers which could predict whether the MPM was in early or advanced stages.[116]

Another controversial serum marker for mesothelioma concerns SV40.[117,118] Links between asbestos exposure and SV40 in human malignant mesothelioma have been demonstrated,[114,119,120] but other studies have been unable to identify such a link.[121–123] A recent study in prediagnostic serum samples was unable to detect the SV40 T antigen, nor was it able to detect SV40 virus DNA.[124] One possible explanation for the disparity concerns contaminated polio vaccines which were linked to the administration of SV40-contaminated polio vaccines from 1954 until 1963.[125] Over the 8 years of use, of 92 million US residents who received polio vaccine, approximately 62% received the potentially SV40-contaminated Salk polio vaccine and of these at least one fifth may have received live, infectious SV40 containing vaccine.[1] Advances in detection methodologies have since led to the suggestion that the conflicting results seen for SV40 in several studies may be due in part to difficulties in accurately detecting SV40-T and/or poor experimental design.[125,126] Indeed there is some evidence that SV40 may be also present in the normal tissues of MPM patients.[127] This would support the clear in vitro evidence demonstrating that SV40 can act as a cocarcinogen in mesothelial cells.[128–130]

Clearly, it is evident that further testing or refinements to existing detection methods and substantial basic research will be required to determine if SV40 T antigen plays an important role in MPM malignancy and if tests to detect SV40 T will prove suitable for development as an MPM biomarker.

Overall, with the identification of new serum biomarkers it may be possible in the future to combine them all into an array based serum test for mesothelioma.

Conclusions and Future Directions

Issues of Array Reliability/Overlap

One of the problems identified with the use of array technology in MPM, has been the lack of concordance between the various predictive or prognostic studies. If one compares all the predictive sets identified,[60–62,64,65] there is very little overlap (Figure 1). From this one can conclude that microarray analysis of MPM would appear to be very "noisy", and the prognostic genes identified should be reevaluated individually to test for prognostic significance. Nevertheless, several of the genes identified in these predictive array studies have also shown up in other MPM array studies. The predictive genes for which some form of alternative array based validation exists are listed in Table 1.

Combined meta-analysis of all available datasets could therefore potentially identify critical genes which are involved in MPM pathogenesis. Several of the published mesothelioma gene expression array studies have been submitted to Oncomine. This research platform suite combines a rapidly growing compendium of cancer transcriptome profiles with a sophisticated analysis engine and a powerful web application for data-mining and visualiza-

tion.[131] Meta-analysis of the mesothelioma studies may provide more robust prognostic genes which could be subsequently validated.

Mesothelioma and the IGF-Axis

One emerging subset of genes which has been shown to be altered in mesothelioma concerns the insulin-like growth factor axis.[132] With microarray analysis, several members of this axis have also been shown to be altered confirming the importance of this pathway in mesothelioma. Two commonly down-regulated genes from the IGF-axis in mesothelioma are *IGFB-4* and *IGFBP*-5.[35,42,62,133] Commonly overexpressed genes include *IGFBP-2*, *IGFBP-3*, *IGFBP-7/MAC25* and an exon specific isoform of IGF-I (IGF-I, exon 1A).[60,66,68,70]

Increased *IGFBP-5* expression has been linked to long term survival (>12 months postoperation).[62] However, in a separate study down-regulation of IGFBP-5 was observed to be a common feature in mesothelioma, but no survival data is available for these samples.[66] It must be noted that *IGFBP-3* is another gene whose overexpression has also been linked to poor outcome tumors,[60] indicating that a comprehensive analysis of the IGFBP family may have clinical value in MPM.

Kratzke and coworkers[66] noted the dysregulation of the IGF-axis in an early microarray study, and reanalysed their microarray data specifically for members of the IGF-axis. They subsequently identified additional genes which were overexpressed in mesotheliomas (*IGF1R*, *IRS-2*, *IGFBP-3*, and *IGFBP-6*).[134] IGF1R was also identified as a gene with altered expression in mesothelioma by Albeda and coworkers.[67] The analysis by Hoang et al.,[134] led to the discovery that there is selective activation of the Insulin receptor substrates -1 and -2 (IRS-1, IRS-2) associated with a distinct subset of MPMs.

Mesothelioma as a Target for Epigenetic Therapies

From the results of the various microarray experiments several genes identified as being altered in mesothelioma have either been associated with chromatin remodelling complexes, or respond to treatment with inhibitors targeting the epigenetic machinery. In particular, CRI1 (CREBBP/EP300 inhibitory protein 1, also known as EID-1) is a CREB-binding protein identified as a significant ($p = 2.7 \times 10^{-4}$) potential mesothelioma tumor marker in the study by Gordon et al.,[70] This protein has been shown to antagonize the action of pRb, p300, and CBP histone acetyltransferase activity.[70] Several cell line studies have also shown that targeting histone deacetylases can sensitize mesothelioma cells to therapeutic approaches.[135–138]

From our evaluation of the available published microarray gene lists, several genes have been shown to be altered in cells following treatment with histone deacetylase inhibitors, and a sample of these is provided in Table 3.

From these indicators, it is clear that histone deacetylase inhibitors represent a potentially new treatment modality in MPM. Early clinical trials of Zolinza (suberoylanilide hydroxamic acid) in mesothelioma demonstrated patient responses.[139,140] These encouraging responses have led to the initiation of a phase clinical III trial of Zolinza in mesothelioma.[141]

Methylation is another epigenetic modification which is frequently associated with the down-regulation of gene expression in MPM.[142–145] A recent study examined the promoter methylation of six cell cycle control pathway genes (*APC, CCND2, CDKN2A*, *CDKN2B*, *HPPBP1*, and *RASSF1*) in a series of 70 MPMs. Significantly higher lung asbestos body burden occurred if any of these cell cycle genes were methylated ($p < 0.02$), and there was a significant trend of increasing asbestos body counts as the number of methylated cell cycle pathway genes increased from 0 to 1, to >1, ($p < 0.005$), and remained significant after controlling for age, gender, and tumor histology.[146] These data suggest that following asbestos exposure aberrant DNA methylation may be the trigger for the clinical course of malignant mesothelioma.

A phase I clinical trial of Decitabine (a DNA methyltransferase inhibitor) in patients which included mesothelioma has recently been completed, and in 2/7 mesothelioma patients stable disease was achieved.[75]

Indeed the importance of therapies targeting epigenetic pathways is actively being explored in mesothelioam research. From a query of the clinical trials database at the ClinicalTrials website (http://clinicaltrials.gov), several phase I/II clinical trials for both histone deacetylase inhibitors and DNA methyltransferase inhibitors involving mesothelioma were identified (Table 4).

Mesothelioma—Consistency for Progression to More Deadly Profiles

Are there any overall features emerging from these studies? If we begin by looking at the responses to cells exposed to fibers patterns emerge indicating pathways for the progression of these cells into more aggressive forms. Expo-

TABLE 3. Genes Identified in MPM Which Respond to HDAC Inhibition

Gene	Array (Change)	HDAC Inhibitor Response	Reference
COL1A1	Elevated	Down-regulated	70,152
Survivin	Elevated	Down-regulation/degradation	151,153–155
bFGF	Elevated in sarcomatoid	Down-regulated	66,156
VEGFR2	Down-regulated	Up-regulated	35 "Gray and O'Byrne, unpublished data"
Cyclin D2	Down-regulated	Up-regulated	35,157

bFGF, basic Fibroblast growth factor; VEGFR, vascular endothelial growth factor receptor; MPM, malignant pleural mesothelioma; HDAC, histone deacetylase; *COL1A1*, collagen type 1 alpha-1.

TABLE 4. Clinical Trials Involving Epigenetic Therapies in Mesothelioma

Company	Intervention	Phase	Clinical Trial Identifier[a]
Novartis	Drug: LBH 589	I	NCT00535951
California Cancer Consortium	Drug: Belinostat (PXD101)	II	NCT00365053
CuraGen Corporation TopoTarget A/S	Drug: Belinostat Drug: 5-Fluorouracil (5-FU)	I	NCT00413322
CuraGen Corporation TopoTarget A/S	Drug: Belinostat	I	NCT00413075
Merck	SAHA (Zolinza/Vorinostat)	II	NCT00128102
Merck	SAHA (Zolinza/Vorinostat)	I	NCT00106626
European lung cancer working party	Drug: Valproate plus doxorubicin	II	NCT00634205
National Cancer Institute (NCI)	Drug: Decitabine	I	NCT00019825
National Cancer Institute (NCI)	Drug: Celecoxib Drug: Decitabine Drug: Romidepsin	I	NCT00041158
Columbia University Schering-Plough Pharmion	Drug: Azacitidine in combination with Temozolomide	I	NCT00629343
National Cancer Institute (NCI)	Drug: Alvocidib Drug: Romidepsin	I	NCT00098644
National Cancer Institute (NCI)	Drug: HDAC inhibitor SNDX-275 (MS-275)	I	NCT00020579
National Cancer Institute (NCI)	Prospective study	N/A	NCT00447447

[a] As found on clinicaltrials.gov (http://clinicaltrials.gov).
NCI, National Cancer Institute; 5-FU, 5-Fluorouracil; N/A, not applicable.

sure to fibers causes the up-regulation of antiapoptotic, proangiogenic, proinflammatory, and prosurvival pathways coupled with the suppression of pathways linked to cell mediated immunity. Are these pathways also affected in other array studies? Antiapoptotic pathways have been shown to be affected in several independent studies.[35,70,74,133,147] Prosurvival pathways have been shown to be affected in several studies,[38,64,69] while cell mediated immunity,[41] proangiogenic,[70] and proinflammatory[62] pathways have also been observed to be altered.

Final Comments

What if anything, can we conclude from the plethora of data on MPM emerging from array based technologies? Clearly, there is an implicit gain in the identification of novel potential prognostic or biomarkers, which can individually be worked up for independent validation. The concern for many researchers and regulatory bodies would be the lack of concordance between studies. Nevertheless continued meta-analysis of the already generated data along with any new data may prove fruitful, or the development of a concerted multicenter study to conduct large scale microarray or proteomic analysis of a defined set of mesothelioma samples.

An additional avenue of approach may be to collate and subject all the currently available data to a "systems biology" analysis,[148] to identify networks of MPM interactions/pathways which might allow for the identification of new therapeutic approaches to the treatment of MPM.

Recently, suggestions have been made to develop a Mesothelioma Virtual Tissue Bank.[149,150] These initial suggestions have centered on the need for "Common Data Elements" in relation to the annotation of samples in biorepositories. A multitiered approach to enhance this could be achieved by incorporating and linking samples to other datasets such as those stored in Oncomine.[131] If further mesothelioma databases were to emerge (e.g., proteomic studies etc), which could be linked into a central virtual tissue bank, combined meta-analysis may allow researchers to quickly identify important targets/candidate genes in mesothelioma. The continued paucity of treatment options with good clinical outcome for MPM means that significant research remains to discover biomarkers for the early detection of MPM, and the identification and validation of druggable targets or prognostic indicators for the treatment of MPM. Array technology represents a unique way to maximize such discoveries. Combinations of array techniques to combine both gene expression and protein profiling may help to narrow down such potential targets.

This review has described how array technology has revolutionized our current knowledge of mesothelioma but much clearly remains to be done to improve the diagnosis and management of this disease.

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